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The remaining studies for this project were to be performed in primary and secondary cultures of rat mammary epithelial cells (RMEC) prepared from ACI and Sprague-Dawley rats. Eight attempts were made to grow these cells, either from freshly prepared cell suspensions or from cryopreserved cells. None of these attempts were successful. Cells began to exhibit an epithelial morphology but within 24-48 hours they ceased growth and detached from the collagen substratum. None of the cells were acceptable for studies either of estradiol metabolism or of the effects of estrogens on mitotic microtubules. This failure to grow these cells prevented the performance of the remaining studies. We recently have acquired spontaneously immortalized lines of RMEC from the estrogen-sensitive ACI hybrid strain and from the estrogen-resistant Copenhagen strain, one of the parental strains of the ACI. Our plan for the remaining funding period is to pursue our aims related to mitotic microtubules and the induction of aneuploidy using these cell lines. Although they are presumed to no longer express cytochrome P450s, and thus cannot metabolize estradiol itself, we nonetheless can treat these cells with estradiol and various metabolites, particularly the catechol estrogens, to determine the ability of these compounds to alter mitosis.

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FOREWORD

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Thomas A Reed 7/14/00

PI - Signature Date

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Introduction

Estrogen exposure represents one of the few clear risk factors for breast cancer in humans. This potential risk is underscored by the finding that both natural and synthetic estrogens are mammary carcinogens in animal models. Despite this clear association between estrogens and breast cancer, the mechanism of tumorigenesis by these agents has not been established. This project will test the hypothesis that estrogens or their metabolites, via covalent binding, are specific disruptors of mitotic microtubules in mammary epithelial cells, and that the resulting interference with mitosis causes aneuploidy. Moreover, the induction of non-random changes in ploidy represents a key step in the malignant transformation of mammary epithelial cells. To test this hypothesis, we will study the metabolism and effects of the mammary carcinogen estradiol (E2), in liver microsomes and in rat mammary epithelial cell lines (RMEC). Microsomes and mammary epithelial cells will be isolated from female ACI, Sprague-Dawley, and Copenhagen (COP) rats. The former strain is extremely sensitive to estrogens as mammary carcinogens, whereas the Sprague-Dawley and COP strains are relatively resistant to estrogens. The concentration- and time-dependence of metabolism and the covalent modification of proteins will be determined for E2 in preparations from each rat strain, as will the specific covalent modification of tubulin subunits. These chemical endpoints will be compared with the determinations of several cellular changes in response to each of the compounds. Effects on cell proliferation will be assessed by immunohistochemical analysis of the proliferation-specific nuclear antigen PCNA (19A2). Effects on the integrity of microtubules (mitotic spindles) also will be determined immunohistochemically using anti-rat tubulin antibodies. Finally, aneuploidy will be analyzed by flow cytometry. These studies of the effects of the compounds on subcellular structure and function constitute a step-wise examination of the proposed mechanism of action for carcinogenic estrogens. Induction of proliferation will be used as a general index of estrogenicity. The effects on microtubules, however, begin to explore the link between covalent modification of tubulin in a target cell for carcinogenicity of these compounds and the disruption of mitosis. If the covalent modification of tubulin, the disruption of microtubules, and the induction of aneuploidy follow the same time- and concentration-dependence then a strong mechanistic link will be forged. This will support future studies on the induction of aneuploidy by estrogens in mammary epithelial cells *in vivo*.

Body

We have shown previously that liver microsomal preparations from female ACI and Sprague-Dawley rats produce dramatically different oxidative metabolites of E2 (1). Specifically, at low E2 concentrations, ACI rat liver produces primarily 4-hydroxy-E2, the presumed carcinogenic metabolite of E2. Sprague-Dawley liver preparations, under the same conditions, produce little if any detectable 4-hydroxy-E2 and instead produce 2-hydroxy-E2, a non-carcinogenic metabolite. Thus the specific products formed by liver microsomes from the two strains fall in line with their relative susceptibilities to E2-induced mammary tumors-- the sensitive ACI strain produces the active carcinogen while the resistant Sprague-Dawley strain produces the non-carcinogenic product. The goal of the project was to move on to the characterization of target-tissue metabolism of E2 by the two strains, using primary and secondary cultures of mammary epithelial cells. This step has been unsuccessful. We have isolated mammary epithelial cells from young female ACI and Sprague-Dawley rats on eight occasions. These cells have been cultured in 100 mm dishes coated with rat tail collagen, using DMEM/F12 medium with 10% serum. These conditions are used routinely in this institution for the culture of mouse mammary epithelial cells (2).

Unfortunately, we were unable to achieve sufficient viability and growth of RMEC to support our studies. We tried eight separate fresh cell preparations, and three separate trials with cryopreserved cells. Although 100 mm plates were seeded with 10^6 cells, and initial growth was observed, the plates never exceeded about 60% confluence, and by that time many dead and dying cells were observed. Without this stable cell population the proposed studies of E2 metabolism and effects could not be performed.

Recently we have learned of a related model system which we will employ for the remainder of the funding period. The laboratory of Jim Shull at the Eppley Cancer Center in Omaha has isolated and characterized spontaneously immortalized non-transformed epithelial cell lines from the mammary glands of the ACI rat and from its' E2-resistant parental strain, the Copenhagen rat (COP). These cells grow readily in low- or no-serum media and retain much of their epithelial morphology and behavior. We propose to use this established, robust cell system for our remaining studies.

The use of the mammary epithelial cell lines retains the essential feature of the original experimental design, namely to study estrogens in target cell populations from strains sensitive to and resistant to the carcinogenic effects of estrogens. In fact, using RMEC derived from the Copenhagen rat, an estrogen-resistant parental strain for the ACI hybrid, we have arguably a closer match between the two strains with the exception of the marked difference in response to estrogens, and thus a cleaner system for sorting out key mechanistic steps related to carcinogenesis. The use of the cell lines also clearly will bypass our failure to produce viable early passage cultures of these cells. The major drawback to the use of these cell lines is the assumption that, like virtually all epithelial cells, they will have ceased expression of cytochrome P450s after so many passages. We will experimentally verify this by examination of E2 metabolism, but we also will press ahead with investigations of how cells from the two strains respond to E2 and its metabolites.

First, we will assess the proliferative effect of E2, estrone (E1), estriol (E3), 4-hydroxy-E2, 2-hydroxy-E2, 4-hydroxy-E1, 2-hydroxy-E1, 4-methoxy-E2, and 2-methoxy-E2 on these cells. Cultures in serum-free medium (3) will be treated with each of these estrogens at concentrations between 0.1 and 10,000 nM. Cell samples will be taken at 6, 12, 24, 48, and 72 hrs and viable cell numbers estimated by tetrazolium dye reduction to determine the effects of each steroid on proliferation rate. These findings will be confirmed at key concentrations and time points by immunohistochemical determination of proliferative fraction, using the 19A2 nuclear antigen as the indicator of proliferation (4).

The next series of experiments will utilize anti-tubulin antibodies to characterize the effects of these estrogens on cytoskeletal structures, particularly the mitotic microtubules (5). Cells will be treated with the estrogens over the same concentration range as noted above, and cells then will be fixed and stained with fluorescent anti-tubulin. A second staining with anti-actin will discriminate between effects on microtubules and more generalized anti-cytoskeletal effects (5). Obviously anti-mitotic effects only may be seen in cells which are actively proliferating. If the proliferative fraction of cells is too low, we will employ hydroxyurea treatment to synchronize cells, and then release them immediately prior to addition of the appropriate estrogen.

Finally, we will examine the ploidy of control and estrogen-treated RMEC using flow cytometry. The original experimental design proposed to examine ploidy and chromosomal aberrations using whole chromosome painting with various fluorescent probes, analyzed using the Vysis computerized imaging system. This approach has been replaced for two reasons. The first is the short time left during this award, which would preclude learning and validating this approach. Second, the fluorescent oligonucleotide probe set for the rat genome, required for this analysis, has not yet been developed. This was to have proceeded to completion prior to its required use in this project, but that has not yet occurred.

Key Research Accomplishments

Primary culture of RMEC from ACI and Sprague-Dawley rats was not successful.

Reportable Outcomes

None.

Conclusions

The primary cell culture system which was to be employed for these studies was not successfully established. This has led to the identification of a substitute system, immortalized cell lines derived from RMEC of ACI and COP rats, which will be employed in the final year of this project. While these cell lines probably will not support the study of E2 metabolism, they will allow the addressing of the effects of E2 and E2 metabolites on cell function, which arguably is a more central mechanistic step in the carcinogenic response to estrogens.

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5. Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., and Mitchison, T.J. (1999) Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* **286**: 971-974.

Statement of Work

It is obvious that due to the problems faced with the original cell culture model and the lack of success with that model, this project has fallen well behind schedule. On the following pages are the original Statement of Work (p. 8) and the **requested** modified Staement of Work (p. 9) for the remainder of the funding period.

STATUS OF PREVIOUS STATEMENT OF WORK

Aim 1.

Task 1. Establish radiometric HPLC assay for estradiol (E2) metabolism	Done
Task 2. Establish radiometric HPLC assay for 7,12-dimethylbenz[a]anthracene (DMBA) metabolism	Done
Task 3. Characterize E2 metabolism in ACI and Sprague-Dawley (SD) rat liver microsomes (RLM)	Done
Task 4. Characterize DMBA metabolism by ACI and SD RLM	Months 25 - 26
Task 5. Optimize culture conditions for ACI and SD rat mammary epithelial cells (RMEC)	Unsuccessful
Task 6. Characterize E2 metabolism by RMEC from ACI and SD rats	Delayed
Task 7. Characterize DMBA metabolism by ACI and SD RMEC	Delayed
Task 8. Characterize covalent modification of ACI and SD RMEC proteins by E2 and DMBA	Delayed
Task 9. Characterize covalent modification of nuclear DNA in ACI and SD RMEC by E2 and DMBA	Delayed

Aim 2.

Task 1. Optimize immunochemical methods for examination of mitotic microtubule integrity in ACI and SD RMEC	Delayed
Task 2. Determine concentration dependence of effects of E2 and DMBA on microtubule integrity in ACI and SD RMEC	Delayed
Task 3. Determine time dependence of effects of E2 and DMBA on microtubule integrity in ACI and SD RMEC	Month 38

Aim 3.

Task 1. Learn and optimize procedures for the detection of aneuploidy in ACI and SD RMEC	Months 39 - 41
Task 2. Determine the concentration dependence of the effects of E2 and DMBA on ploidy in ACI and SD RMEC	Months 42 - 45
Task 3. Determine the time dependence of the effects of E2 and DMBA on ploidy in ACI and SD RMEC	Months 46 - 48

PROPOSED STATEMENT OF WORK

Aim 1.

Task 1. Establish radiometric HPLC assay for estradiol (E2) metabolism	Done
Task 2. Establish radiometric HPLC assay for 7,12-dimethylbenz[a]anthracene (DMBA) metabolism	Done
Task 3. Characterize E2 metabolism in ACI and SD RLM	Done
Task 4. Characterize DMBA metabolism by ACI and SD RLM	Delete
Task 5. Optimize culture conditions for ACI and COP-derived rat mammary epithelial cell lines (RMEC)	Months 38 - 39
Task 6. Characterize E2 metabolism by RMEC from ACI and SD rats	Delete
Task 7. Characterize DMBA metabolism by ACI and SD RMEC	Delete
Task 8. Characterize covalent modification of ACI and COP RMEC proteins by E2	Months 41 - 45
Task 9. Characterize covalent modification of nuclear DNA in ACI and COP RMEC by E2	Delete

Aim 2.

Task 1. Optimize immunochemical methods for examination of mitotic microtubule integrity in ACI and COP RMEC	Months 40 - 41
Task 2. Determine concentration dependence of effects of E2 on microtubule integrity in ACI and COP RMEC	Months 42 - 44
Task 3. Determine time dependence of effects of E2 on microtubule integrity in ACI and COP RMEC	Month 45

Aim 3.

Task 1. Learn and optimize procedures for detecting aneuploidy in ACI and COP RMEC	Month 46
Task 2. Determine the concentration dependence of the effects of E2 on ploidy in RMEC	Months 47 - 48
Task 3. Determine the time dependence of the effects of E2 on ploidy in RMEC	Months 47 - 48